

## Method for differentiating mesenchymal stem cells into steroid-producing cells

### Field of the Invention

The present invention relates to a method for differentiating mesenchymal stem  
5 cells into steroid-producing cells.

### Prior Art

It is well known that mesenchymal stem cells have multiple differentiation  
potential *in vivo* beyond germ layers (references 1-3). It is expected that the  
10 mesenchymal stem cells will play an important role in the fields of development and  
regeneration, since these stem cells are easily obtained from adults and are easily  
subjected to established cell lines after differentiation in contrast to other embryo stem  
cells. The method for differentiating mesenchymal stem cells into adipocytes,  
chondrocytes or osteoblasts has been examined already (reference 4).

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reference 1: J. Clin. Invest. 10, 697-705, 1999

reference 2: Exp. Cell. Res. 288, 51-9, 2003

reference 3: Biochem. Biophys. Res. Commun. 295, 354-61, 2002

reference 4: WO2002/022788

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### Problems to be solved by the Invention

The present inventors discovered a method for controlling the differentiation of  
mesenchymal stem cells derived from bone marrow into steroid-producing cells. The  
inventors believe that the method of the present invention will become very useful  
25 method in the field of development and regeneration.

### Means to solve the Problems

The present inventors discovered that mouse bone marrow mesenchymal stem  
cells were differentiated into Leydig cells, steroid hormone-producing cells, when  
30 transplanted into testis (Example 4). Furthermore, it was found that a limited portion  
of mouse bone marrow-derived mesenchymal stem cells KUM9 were spontaneously  
differentiated into steroid-producing cells during culturing, and at the same time, that

a transcriptional factor SF-1 was expressed (Example 5). Therefore, the inventors prepared a KUM9-derived strain, wherein SF-1 is expressed constitutively, and added a stimulation by cAMP, then the inventor found that the KUM9-derived cells stably produced steroid hormone (Examples 1 and 2). Moreover, it was confirmed that not only a gonadal steroid hormone-producing enzymes but also adrenal steroid hormone-producing enzymes are induced in a human cell line (Example 3). It is expected that these results are fundamental data in using differentiation of stem cells for medical treatment of human steroid hormone deficiency and provide a useful method for regeneration treatment.

From the above results, the inventors discovered that stimulation of mesenchymal stem cells by a transcriptional factor (SF-1), preferably with SF-1 and cAMP, enabled to differentiate the mesenchymal stem cells into steroid-producing cells and accomplished the present invention.

Namely, the present invention is a method for differentiating bone marrow-derived mesenchymal stem cells into steroid hormone-producing cells, comprising stimulating the mesenchymal stem cells by a transcriptional factor (SF-1). Furthermore, said method may comprise further stimulating the mesenchymal stem cells by cAMP.

The mesenchymal stem cells are preferably derived from bone marrow and said mesenchymal stem cells are preferably human cells.

Still furthermore, the present invention is a method for producing steroid-producing cells, comprising producing steroid producing cells by implementing said method in vitro or by transplanting the mesenchymal stem cells into a mammalian reproductive organ.

Moreover the present invention is steroid-producing cells or non-human animals containing said steroid-producing cells obtained by these method.

#### Advantages of the present invention

The present invention provides, for the first time, a method for differentiating mesenchymal stem cells into steroid hormone-producing cells.

It will be possible to obtain a large amount of differentiated cells by transplanting stem cells into a genital gland of a non-human animal such as swine, if immune difficulties were overcome. The most important issue in regeneration medicine and

others is to maintain transplanted cells keeping the differentiated state and the present invention may be applied as a tool for the development of said method. Said differentiated cells may be used as model cells for the development of regeneration medicine.

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#### Brief Description of the Drawings

Figure 1 shows pIRES-puro2 vector.

Figure 2 shows KUM9 cells (A) and KUM9 cells stably transfected with parent vector (B) and with SF-1 expressing vector (C).

10 Figure 3 shows the western blotting showing SF-1 protein detected in various cell lines. SF-7 and SF-9 are the 7th and 9th cell lines, respectively, which are established by transduction of SF-1.

Figure 4 shows the result of RT-PCR for RNA isolated from KUM9 cells, which are stably transduced with parent vector (pIRES) and SF-1 (SF-9) and are cultured for 7  
15 days in the presence of cAMP. Days shown on the upper row are days until extraction of RNA after the addition of cAMP. HSD3b (3b-HSD) expresses on the 3rd day and P450c17 and P450c11b1 express on the 5th day.

Figure 5 shows the western blotting for proteins extracted from the cells cultured under a condition similar to that described in Fig. 4. Proteins from MA10-derived cells  
20 originated from mouse Leydig cells are used as a positive control.

Figure 6 shows quantification by RIA of secreted steroid hormone, which is obtained by the recovery of supernatant of the medium used for the cells cultured under a condition similar to that described in Fig.4. The amount of hormone produced within 24 hrs was determined by changing culture medium to fresh medium on the day  
25 before the each indicated day.

Figure 7 shows immunostaining by anti-3 $\beta$  HSD I antibody of SF-9 cells, which are fixed before adding cAMP (-) or after culture for 7 days in the presence of cAMP (+). DAPI (4',6-Diamidino-2-phenylindole, dihydrochloride) is a dye marker for DNA and shows the intracellular localization of nucleus.

30 Figure 8 shows the result of RT-PCR for RNA extracted from hMSC E6/E7 cells, which is derived from human bone marrow mesenchymal stem cells, are stably transduced with SF-1, and are cultured for 7 days in the presence of cAMP.

Figure 9 shows testis transplanted with mesenchymal stem cells derived from bone marrow. ST shows seminiferous tubule.

Figure 10 shows pEGFP-1 vector.

Figure 11 shows pPUR vector.

5 Figure 12 shows the charts of flow cytometry for a GFP positive clone.

Figure 13 shows immunostaining of P450scc in GFP positive cells.

Figure 14 shows the results of RT-PCR of various enzymes in GFP positive (scc+) and negative (scc-) cell fractions.

## 10 Detailed Description of the Invention

Mesenchymal stem cells are derived from various interstitial cells and are able to differentiate into various cells. Mesenchymal stem cells are present not only in bone marrow but also interstitial and other tissues (e.g. in kidney, articular membrane, amnion and cord blood) of mesoderm-derived tissues. All these mesenchymal stem  
15 cells can be used in the present invention.

Mesenchymal stem cells derived from bone marrow are interstitial cells, which present in marrow stroma and maintain hematopoiesis, and said stem cells from even adult bear pluripotency of differentiating into various cells and tissues. Mesenchymal stem cells derived from bone marrow may be isolated by inserting an injection needle  
20 into an epiphysis for example, by feeding PBS into the epiphysis, by pulling out bone marrow cells, by seeding and subjecting the bone marrow cells to adhere to a plastic dish and by repeating the subculture.

The transcriptional factor (SF-1), an inducing factor used in the method of the present invention, is an orphan intranuclear receptor, which is expressed in genital  
25 and adrenal gland-type steroid hormone-producing cells, and has been known to control the transcription of steroid hormone producing-enzymes (Endocrine Reviews vol.18, No.3, 361-377 (1997); The FASEB Journal vol.10 1569-1577 (1996)). Even if SF-1 is derived from different animal species, SF-1 binds to a common target DNA sequence in mesenchymal stem cells and the factor is expected to provide the same  
30 result.

cAMP exists ubiquitously in all living organism, whose intra-cellular concentration is  $10^{-6}$  to  $10^{-7}$  M. cAMP participates in generation of specific enzymes and metabolic

control in target cells and also participates in growth and differentiation of cells. cAMP is a second messenger of LH and ACTH, which induces the expression of steroid hormone production-related enzymes in genital and adrenal glands and enhances the production of steroid hormones.

5       The mean to stimulate by the transcriptional factor (SF-1) and cAMP may include the direct contact of these factors with mesenchymal stem cells or the use of a vector expressing these factors.

      To differentiate mesenchymal stem cells into steroid hormone-producing cells, mesenchymal stem cells may be stimulated by an inducing factor in vitro or said cells  
10       may be transplanted into a mammalian reproductive organ. In the case of in vitro induction, for example, human mesenchymal stem cells may be cultured in DMEM (Dulbecco's Modified Eagle's Medium) containing 10% FBS and mouse stem cells may be cultured in IMDM (Iscove's Modified Dulbecco's Medium) or in DMEM containing 10% FBS. The above cells are cultured in an incubator with 5% CO<sub>2</sub> at 37°C as usual.  
15       The concentration of SF-1 is about 0.1~10 µg, preferably at 1 µg / 1x10<sup>5</sup> cells, and that of cAMP is about 0.5~2.0 mM, preferably at 1 mM / 1x10<sup>5</sup> cells.

      When mesenchymal stem cells were transplanted into a mammalian reproductive organ, a certain factor activates the cell (in the case of organ transplantation, interaction with the surrounding cells) to express SF-1, which is considered to make  
20       these stem cells to differentiate into steroid hormone-producing cells.

      In the differentiation of mesenchymal stem cells, the role of cAMP is subsidiary. Furthermore, since cAMP is necessarily present in all kind of cells with a large variety of concentrations, intracellular concentration of cAMP might be raised by some reason without adding exogenous cAMP.

25       As described above, steroid-producing cells may be obtained accordingly, then steroid hormone may be obtained from the steroid-producing cells.

      Steroid-producing cells derived from mesenchymal stem cells include adrenal cortical cells, ovarian granulosa cells, ovarian capsular cells, testis Leydig cells, testis, Sertoli cells and others.

30       Obtained steroid hormones include any steroid hormone and the like, which are synthesized from cholesterol, such as progestin, androgen, estrogen, glucocorticoid and mineralcorticoid.

The following examples further illustrate the present invention, but it is not intended to limit the scope of the present invention.

Gene expression of steroid hormone synthesizing enzymes was examined by RT-PCR using the primers shown in Table 1.

Table 1

Symbol	Enzyme	Primer	SEQ ID NO:
StAR	steroidogenic acute regulatory protein	F-GAAGGAAAGCCAGCAGGAGAACG	1
		R-CTCTGATGACACCACTCTGCTCC	2
P450scc	cholesterol side chain cleavage enzyme	F-CGCACAGTCCAGAACAACAAGCA	3
		R-CGGTTAGAGAAGGCAGGATAGAG	4
HSD3b	3- $\beta$ hydroxysteroid dehydrogenase	F-GCAGACCATCCTAGATGTCAAT	5
		R-TCATCATAGCTTTGGTGAGG	6
P450c17	17- $\alpha$ -hydroxylase/17,20-lyase	F-AAAATAATAACACTGGGGAAGGC	7
		R-TGGGTGTGGGTGTAATGAGATGG	8
P450c21	steroid 21-hydroxylase	F-AGAGGATCCGCTTGGGGCTGC	9
		R-GGAGAATTCTTATGGATGGC	10
P45011b1	steroid 11- $\beta$ -hydroxylase	F-TCACCAAATGTATCAAGAATGTGT	11
		R-CCATCTGCACATCCTCTTCTCTT	12
P45011b2	aldosterone synthase	F-CCAACAGATGTATCTGGAAGGTGC	13
		R-CCATCTGCACATCCTCTGCCTCA	14
GAPDH	glyceraldehyde phosphate dehydrogenase	F-ACCACAGTCCATGCCATCAC	15
		R-TCCACCACCCTGTTGCTGTA	16

#### Example 1

The coding region (Nucleotides 1~1389 of SEQ ID NO: 17) of rat SF-1 gene (Gene Bank NM\_053344, SEQ ID NO:17) was integrated in pIRES-puro2 vector (Clontech, Fig. 1). KUM9 cells (obtained from Department of Reproductive Biology, National Research Institute for Child Health and Development), an established adult mouse-derived mesenchymal stem cell line, were transfected with said vector after linearized with Nru I and were selected in a medium containing puromycin (2  $\mu$ g/ml) at 48 hrs after the transfection. The resistant colonies remained for 2 weeks in the medium were picked up and were cloned.

Those clones transfected with parent vector pIRES-puro (Fig. 2B) showed similar morphology to that of KUM9 without transfection (Fig. 2A). In contrast, a large number of fat droplets, conceivably containing cholesterol, i.e. a precursor of steroid hormones, were observed intracellularly in clones (SF-7 and SF-9) expressing SF-1 (Fig.

2C).

Proteins were extracted from each clone (SF-7 and SF-9), were examined the expression of SF-1 protein by Western blotting and SF-1 protein was detected (Fig. 3).

Furthermore, gene expression of steroid hormone synthesizing enzymes in these  
5 cells was examined by RT-PCR and the expression of P450scc was confirmed.

### Example 2

The cloned cells (SF-9), wherein expression of SF-1 was observed in example 1, were cultured for 7 days in the presence of cAMP (1 mM, Sigma). On 1,3,5 and 7 days  
10 after addition of cAMP, RNA was extracted from said cells and was examined the gene expression by RT-PCR. For comparison, the same procedures were performed for cloned cells using the parent vector pIRES as a negative control.

The results show that the expression of p450scc was induced after 1 day, HSD3b1 was induced after 3 days and furthermore p450c17 was induced after 5 days (Fig. 4).

15 The result of Western blotting shows that the expression of these enzyme proteins was induced similar to that of mRNA (Fig. 5).

Moreover, according to these results, those cells without transfection with SF-1 (pIRES-puro) do not express steroid hormone-producing enzymes by the addition of cAMP and do not produce steroid hormone. These facts could be interpreted as that  
20 only cAMP could not induce differentiation into steroid hormone-producing cells.

The interpretation depicts that SF-1 is an essential factor to differentiate mesenchymal stem cells into steroid hormone producing cells and cAMP is a cofactor.

The output of steroid hormone was measured by radioimmuno assay (RIA) after recovery of culture medium on the 3rd day and 7th day. According to the results,  
25 progesterone and androgen were detected on the 3rd day and 7th day, respectively, in parallel to the results of expression of mRNA and proteins (Fig.6) in the group of cells added with cAMP. Not only the induction of steroid synthesizing enzyme but also the synthesis of androstendione, a kind of androgenic hormone, was confirmed.

Immunostaining of HSD3b1 shows that all cells are positive (Fig. 7).

30 The results of examples 1 and 2 show that forced expression of SF-1 leads mesenchymal stem cells derived from bone marrow to differentiate into steroid hormone-producing cells.

### Example 3

Induction of steroid hormone-producing cells for human cells was examined in an experiment similar to example 2 using human derived cells. Human bone marrow mesenchymal stem cells hMSCE6/E7 (gifted from Field of Clinical Application, Institute for frontier Medical Sciences, Kyoto University) were transfected with said pIRES-puro-SF-1 vector by calcium phosphate method. At 48 hrs after transfection, cells were selected in a medium containing puromycin (0.3 µg/ml), puromycin resistant colonies were picked up after 2 weeks and they were cloned. After 7 days culture in the presence of cAMP, RNA was isolated and gene expression was examined by RT-PCR.

hMSC cells stably expressing pIRES-puro-SF-1 did not show morphological change as observed for KUM9 cells (Fig. 2A~C). However, analysis of gene expression by RT-PCR shows the induction of expression of a group of steroid hormone synthesizing genes such as StaR, P450scc and 3β-HSD. Furthermore, addition of cAMP induced the expression of P450 c21 and P450 11b1, glucocorticoid producing enzymes in adrenal cortex (Fig. 8).

These results show that human bone marrow-derived mesenchymal stem cells were differentiated into steroid hormone-producing cells.

### Example 4

In this example, bone marrow-derived mesenchymal stem cells were transplanted into testis. Bone marrow cells were isolated from epiphysial region of os longum from limbs of 5 w.o. male Green rat by insertion of a 26-gauge needle for syringe and by filling of PBS. After red blood cells were disrupted by osmotic shock, said cells were seeded at  $5 \times 10^7$  / 100 ml dish containing α-MEM medium fortified with 20% FBS. After 24 hrs, the medium was changed to remove non-adherent cells. After 3 days, cells were trypsinized by 0.25% trypsin, subcultured, and cultured for further 1 week. These procedures lead to concentrate mesenchymal stem cells among various bone marrow-derived cells. After confluent cells were trypsinized,  $1 \times 10^6$  cells were suspended in 50 µl PBS and were injected into testis of 3 w.o. male Sprague Dawley rat (Japan Charles River Co.).

After 3 weeks, the testis was removed, observed under inverted microscopy and

fixed by 4% para-formaldehyde. Frozen sections were prepared and used for immunohistochemical method. Double staining was performed using rabbit anti-rat P450scc antibody (CHEMICON) and mouse anti-GFP antibody (MBL) for the first antibody and Cy3-conjugated anti-rabbit IgG antibody (SIGMA) and FITC-conjugated anti-mouse IgG (SIGMA) as the second antibody.

GFP positive cells, possibly derived from Green rat bone marrow, were observed easily under inverted fluorescent microscopy and were detected in testis at 3 weeks after transplantation (Fig. 9A). Therefore, it was found that transplanted cells adhered to host testis. To examine whether these cells were differentiated into steroid hormone producing cells (Leydig cells), double immunostaining using anti-GFP (Fig. 9B) and anti-P450scc (Fig. 9C) was performed. Since most of P450scc positive cells were also GFP positive, Green rat bone marrow-derived cells were found to be differentiated into Leydig cells.

#### Example 5

Stem cells could not completely be cloned by the method of example 4 because of the contamination of macrophage cells. Therefore, an experiment was performed to examine whether KUM9 cells, an established adult mouse-derived mesenchymal stem cells, has a potency to be differentiated in vitro into steroid hormone-producing cells.

P450scc (a side chain cleavage enzyme) is an enzyme, which is localized on mitochondrial inner membrane and catalyzes a reaction degrading cholesterol into pregnenolone. Since the above reaction is a necessary procedure in all steroid hormone production, P450scc is expressed in all steroid hormone-producing cells.

The group of Chung et al. prepared and used transgenic mice with a visual reporter gene Lac Z and showed that 2.3 Kbp of 5' upstream region in human P450scc gene (Gene Bank M60421) controls the expression of P450scc gene in steroid hormone-producing cells in genital and adrenal gland (Endocrinology, 140:5609-5618, 1999). Therefore, preparation of stem cells, wherein the visual reporter gene expresses by the control of the said 5' upstream region, conceivably enables to detect the differentiation into steroid hormone-producing cells.

Therefore, P450scc gene (Gene Bank M60421, SEQ ID NO: 18) was integrated into the upstream of EGFP protein coding region of pEGFP-1 vector (Fig. 10, Clontech) and

the vector was fused with pPUR vector (Fig. 11, Clontech) coding puromycin resistant gene.

Said synthetic vector, after linearized with ApaL I, was transfected into KUM9 cells (Gifted from Department of Reproductive Biology, National Research Institute for Child Health and Development), derived from mouse bone marrow mesenchymal stem cells by lipofection. Since fluorescent GFP expresses at the time of expression of P450scc in these cells, spontaneously differentiated KUM9-derived steroid-producing cells could be separated by flow cytometer using fluorescence as a probe.

At 48 hrs after the separation, cells were selected in the medium containing puromycin (2 µg/ml) and resistant colonies after 2 weeks was cloned by a limiting dilution method.

GFP positive cells were detected in 2 clones out of isolated 250 clones during growing phase of these cells under inverted fluorescence microscopic observation.

GFP positive and negative cells were separated by flow cytometer for said 2 clones, wherein a lot of GFP positive cells were found, and GFP positive cells were 2~5% (Fig. 12).

Furthermore, immunostaining of P450scc for GFP positive cells confirmed that GFP positive cells were also P450scc positive (Fig. 13).

Moreover, GFP positive (scc+) and negative (scc-) cells were separated by a cell sorter, total RNA was extracted from the separated cells and cDNA was prepared by reverse transcription reaction using reverse transcriptase. RT-PCR was performed using a specific primer for each gene. The expression of mRNA of P450scc gene was observed in GFP positive cells (scc+) (Fig 14). Additionally, the transcriptional factor SF-1 was expressed in said cells isolated. Still furthermore, examination of the expression of genes involved in steroid hormone production revealed that the expression of 3β-HSD gene of both genital and adrenal gland type (HSD3b1) and placental type (HSD3b6) was observed.

From these results, it was interpreted that the transplantation induced the expression of SF-1 in stem cells, and that these stem cells differentiated into steroid hormone-producing cells on contact with SF-1.